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Recognition of IgG by Fcγ Receptor

THE ROLE OF Fc GLYCOSYLATION AND THE BINDING OF PEPTIDE INHIBITORS*

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Recently determined crystal structures of the complex between immunoglobulin constant regions (Fc) and their Fc-respective receptors (FcR) have revealed the detailed molecular interactions of this receptor-ligand pair. Of particular interest is the contribution of a glycosylation at Asn^{297} of the C_H2 domain of IgG to receptor recognition. The carbohydrate moieties are found outside the receptor·Fc interface in all receptor·Fc complex structures. To understand the role of glycosylation in FcR recognition, the receptor affinities of a deglycosylated IgG1 and its Fc fragment were determined by solution binding studies using surface plasmon resonance. The removal of carbohydrates resulted in a non-detectable receptor binding to the Fc alone and a 15- to 20-fold reduction of the receptor binding to IgG1, suggesting that the carbohydrates are important in the function of the FcγRIII. Structurally, the carbohydrates attached to Asn^{297} fill the cavity between the C_H2 domains of Fc functioning equivalently as a hydrophobic core. This may stabilize a favorable lower hinge conformation for the receptor binding. The structure of the complex also revealed the dominance of the lower hinge region in receptor·Fc recognition. To evaluate the potential of designing small molecular ligands to inhibit the receptor function, four lower hinge peptides were investigated for their ability to bind to the receptor FcγRIII. These peptides bind specifically to FcγRIII with affinities 20- to 100-fold lower than IgG1 and are able to compete with Fc in receptor binding. The results of peptide binding illustrate new ways of designing therapeutic compounds to block Fc receptor activation.

Activation of low affinity Fcγ receptors requires the binding of antigen-activated immune complexes (1, 2). The recent determination of crystal structures of Fcγ and Fcε receptors in complexes with Fc fragments have revealed that these receptors bind asymmetrically at a 1:1 ratio to the lower hinge region of their dimeric Fc ligands (3–5). This mode of binding is conserved in both FcγRIII and FcεRI.[1] However, a number of questions still remain to be addressed. The first is the role of carbohydrate in the receptor·Fc recognition. In particular, the contribution of a conserved glycosylation site, Asn^{297} of the constant region of IgG1, remains controversial (6–9). The carbohydrate attached to this glycosylation site is partially ordered in all known structures of Fc (10), intact antibody (11), and in the receptor·Fc complexes, suggesting that a stable rather than a flexible conformation exists for the carbohydrate. Furthermore, unlike most glycosylations that attach to surfaces of the molecules, the carbohydrate attached to Fc is located in a cleft between the two C_H2 domains to partially fulfill the cleft. This unique location of carbohydrate may contribute to the conformational stability of Fc, specifically, the relative orientation between the two chains of Fc. Because the receptor epitope is formed primarily by the joint hinge segments of both chains of Fc, receptor recognition is potentially sensitive to the relative orientation of the two C_H2 domains. Thus, it is conceivable that the unique position of the glycosylation at Asn^{297} may contribute to the receptor binding by stabilizing the lower hinge conformation. Although earlier investigations of Fcγ receptor binding to IgG demonstrated a drastic reduction on cell surface receptor affinity when the carbohydrates at Asn^{297} were removed, similar studies on IgE binding to Fcε receptor revealed a much weaker influence of carbohydrate on receptor-ligand recognition (6–9). There are no direct contacts observed between the receptor and the carbohydrate attached to Asn^{297} in the crystal structures of the receptor·Fc complexes to account for the observed effect of carbohydrate on the Fcγ receptor binding.

Certain autoimmune diseases, such as rheumatoid arthritis, result from the activation of Fcγ receptors by auto-antibodies (12, 13). The ability to inhibit the receptor activation in this case should help to control the antibody-mediated auto-inflammatory response. The structures of the receptor·Fc complexes revealed the dominance of the lower hinge residues of Fc in the receptor binding, suggesting a new way of designing small peptide ligands that can inhibit the binding of immunoglobulins to their receptors. These receptor inhibitors may be potential candidates for the treatment of autoimmune diseases.

In addition, the properties that determine the immunoglobulin isotype specificities of the low affinity Fcγ receptors remains to be identified. The isotype specificities of this receptor have been correlated to the pathogenicity of an anti-erythrocyte auto-antibody (14). Earlier results of chimeric IgG work as well as mutational analysis suggest that the lower hinge region is important in determining the receptor preferences (15, 16).

In this work, we demonstrate, using solution binding studies that the carbohydrates attached to Asn^{297} of IgG1 are critical for Fcγ receptor recognition. Synthetic peptides, 8 to 12 residues long and that mimic the lower hinge regions of IgG1, IgG2, IgG4, and IgE, were also shown to bind FcγRIII and to compete with the binding of the native Fc.

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[1] The abbreviations used are: Fc, immunoglobulin constant regions; RcR, Fc receptor; ESI-MS, electrospray ionization-mass spectrometry; SPR, Surface plasmon resonance; NHS, *N*-hydrosuccinimide; EDC, 1-ethyl-3(-3-dimethylaminopropyl)carbodiimide hydrochloride.

TABLE I
Immobilization of the lower hinge peptides and FcγRIII

Sample name	Peptide length	Peptide sequence	Immobilization	
			Concentration	Response
				RU[a]
pIgG1	12-mer	CPAPE**LLGG**PSV	10 mM	295
cIgG1	12-mer	CPAPE**LLGG**PSV	10 mM	586
pIgG2	8-mer	PPVAGPSV	10 mM	362
pIgG4	9-mer	PEFLGGPSV	10 mM	313
pIgE	9-mer	DSNPRGVSA	10 mM	473
pALA	9-mer	AAADAAAAL	10 mM	449
FcγRIII	Flow cell 2		15 μM	10,182
	Flow cell 3		45 μM	11,731

[a] RU, resonance units.

EXPERIMENTAL PROCEDURES

Preparation of Fc by Papain Digestion—The Fc fragment of a human monoclonal IgG1 was isolated by papain digestion (17). In brief, IgG1 at a concentration of 10 mg/ml was incubated for 2 h at 37 °C with 6.6% (w/w) papain at pH 7.1 in the presence of 1 mM cysteamine. This lead to complete cleavage of IgG1. The Fc fragment was separated from the Fab fragments on a Protein A affinity column (Amersham Pharmacia Biotech) with MAPS II (Bio-Rad) binding and elution buffers at a flow rate of 0.5 ml/min. The Fc fragment was further purified on a Superdex 200 HR 10/30 gel filtration column (Amersham Pharmacia Biotech) with 50 mM NaCl, 50 mM Tris at pH 8.0 as a running buffer at a 0.5 ml/min flow rate.

Deglycosylation of Fc—Deglycosylated Fc fragments and IgG1 were prepared by peptide-*N*-glycosidase F (New England BioLabs) digestion in water for 1.5 h at 37 °C using 1 unit of enzyme/10 μg of protein. The extent of deglycosylation was analyzed by SDS-polyacrylamide gel electrophoresis, gel filtration chromatography, and electrospray ionization mass spectrometry (ESI-MS). The gel filtration experiments were carried out using a Superdex 200 PC 3.2/30 gel filtration column (Amersham Pharmacia Biotech) and an Äkta high pressure liquid chromatography purifier (Amersham Pharmacia Biotech) with 50 mM NaCl, 50 mM Tris at pH 8.0 as running buffer at a 0.1 ml/min flow rate. ESI-MS measurements were acquired and recorded with a PerkinElmer Life Sciences Sciex API-300 triple quadruple system (Thornhill, Ontario, Canada) using 8 μg of native or deglycosylated Fc fragment and 6 μg of native or deglycosylated IgG1. The native and deglycosylated IgG1 were reduced with 100 mM dithiothreitol prior mass spectrometry measurements.

Preparation of the Lower Hinge Peptides—Peptides of 8 to 12 amino acids in length with the sequences of the lower hinge receptor binding region of IgG1, IgG2, IgG4, and IgE were synthesized (Table I). All peptides were purified on a Superdex 200 HR16/60 gel filtration column (Amersham Pharmacia Biotech) with H_2O as the running buffer to remove impurities and to exchange the original buffer. A mostly polyalanine peptide (pALA) with a sequence of AAADAAAAL was used as the control. The concentrations of peptides were estimated using absorbance at 220 nm and an extinction coefficient of 2.6 absorbance units per ml mg^{-1}. All peptides were confirmed by mass using ESI-MS. Peptide pIgG1 includes a lower hinge cysteine residue that forms the conserved disulfide bond between the immunoglobulin heavy chains. The disulfide-bonded dimeric form of the peptide was generated by incubating the peptide under a mild base condition of pH 8.0 for 1 h at room temperature. This disulfide-bonded form, resistant to alkylation by iodoacetamide (data not shown), was designated as cIgG1. The free cysteine in the monomeric pIgG1 was blocked by alkylation with iodoacetamide to prevent the dimerization.

Surface Plasmon Resonance Measurements—Surface plasmon resonance (SPR) measurements were performed using BIAcore 2000 instrument (BIAcore AB). FcγRIII receptor was immobilized at concentrations of 15 and 45 μM in 10 mM sodium acetate, pH 6.0, on a CM5 sensor chip using *N*-hydrosuccinimide/1-ethyl-3(-3-dimethylaminopropyl)carbodiimide hydrochloride (NHS/EDC) at a flow rate of 5 μl/min (Table I). Flow cell 4 of every sensor chip was mocked with NHS/EDC as a negative control of binding. All peptides were immobilized at 10 mM concentration on CM5 sensor chips in 100 mM EDTA at pH 8.0 with NHS/EDC at a flow rate of 2 μl/min. The binding buffer consisted of 20 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, 10 mM HEPES at pH 7.4 mixed with various concentrations of analyte. Binding of the native and deglycosylated Fc fragments to the immobilized FcγRIII was measured using serial dilutions of the analyte from 10 to 0.078 μM at a flow rate of 5 μl/min. The binding of IgG1 and its deglycosylated form to the immobilized receptor was measured with the analyte concentrations varying from 10 to 0.02 μM. For the binding of the receptor to immobilized peptides, the analyte consisted of a serial dilution of the receptor between 750 and 0.37 μM. The same immobilized peptide chips were used for competition experiments in which the analyte consisted of 10 μM FcγRIII mixed with various concentrations of Fc from 10 to 0.02 μM. To measure the binding between protein G and Fc, protein G was immobilized on a CM5 sensor chip at 50 μM concentration. The native or the deglycosylated Fc at concentrations between 5 and 0.15 μM were used. All dissociation constants (K_D) were obtained either from a linear regression of steady state 1/Response *versus* 1/C plots using ORIGIN 3.0 (MicroCal Software, Inc.) or from kinetic rate constants fitted with the BIAevaluation software package (BIAcore AB).

RESULTS

Deglycosylation of Fc—Papain digestion of the human IgG1 resulted in a 53-kDa disulfide-bonded Fc fragment. Due to the carbohydrate attachment to Asn^{297}, the mass spectrum of the native Fc fragment displays considerable heterogeneity in mass (Fig. 1*A*). Treatment of the native Fc fragment with peptide-*N*-glycosidase F under non-denaturing condition resulted in a shift of molecular mass of the Fc from 53,400 to 50,345 Da (Fig. 1*B*). This agrees well with the predicted 50,407-Da molecular mass of the polypeptide backbone of Fc, indicating a complete enzymatic removal of carbohydrates. The deglycosylated Fc has an apparent molecular weight similar to that of the native Fc in a Superdex 200 SMART gel filtration column (Amersham Pharmacia Biotech) (Fig. 1*C*). It remains as a disulfide bonded dimer (Fig. 1*D*). The result of a native gel electrophoresis shows that the deglycosylated Fc fragment appears to be more compact than the native Fc (Fig. 1*E*).

Binding of FcγRIII to the Native and Deglycosylated Ligands—Both deglycosylated IgG1 and its Fc fragment were prepared under similar conditions (under "Experimental Procedures") and purified by Superdex 200 HR 10/30 size exclusion chromatography. Solution binding experiments were performed using a BIAcore 2000 instrument with the receptor immobilized on a CM5 sensor chip. The analyte consisted of serial dilutions of IgG1 or Fc with concentrations from 10 to 0.02 μM and 10 to 0.08 μM, respectively. The affinity of FcγRIII for the native Fc fragment was essentially the same as that for IgG1, ~5 μM (Table II). Deglycosylation of the Fc fragment resulted in no detectable receptor binding (Fig. 2*A*), and that of IgG1 resulted in a K_D of ~50 μM, a 10-fold reduction in the receptor binding affinity (Table II, Fig. 2*B*).

To rule out the possibility that the loss of receptor binding was due to a global disruption of the Fc structural fold induced by deglycosylation, binding of both the native and deglycosylated Fc to protein G, which recognizes the C_H2-C_H3 junction region of Fc, were also measured on a immobilized protein G sensor chip. The protein G binding dissociation constant is 47 nM for the native Fc and 130 nM for the deglycosylated Fc. The ability of deglycosylated Fc to bind protein G suggests no global disruption in the structure upon removal of the carbohydrate. This is also evident from the gel filtration and native gel electrophoresis analyses, both of which show a slightly more compact shape of the deglycosylated Fc compared with that of the native Fc (Fig. 1, *C* and *E*).

Binding of the Lower Hinge Peptides to Immobilized FcγRIII—The crystal structure of FcγRIII in complex with Fc illustrates that the receptor primarily recognizes the lower hinge region of the Fc with 60% of the interface area contributed by the four lower hinge residues, Leu-Leu-Gly-Gly (234–237). To test whether the lower hinge alone can be recognized by the receptor, peptides with the corresponding lower hinge sequences of IgG1/3, IgG2, IgG4, and IgE were synthesized (Table I). All four peptides were able to bind to the immobilized receptor on a CM5 sensor chip (Fig. 3), although pIgG1 binds

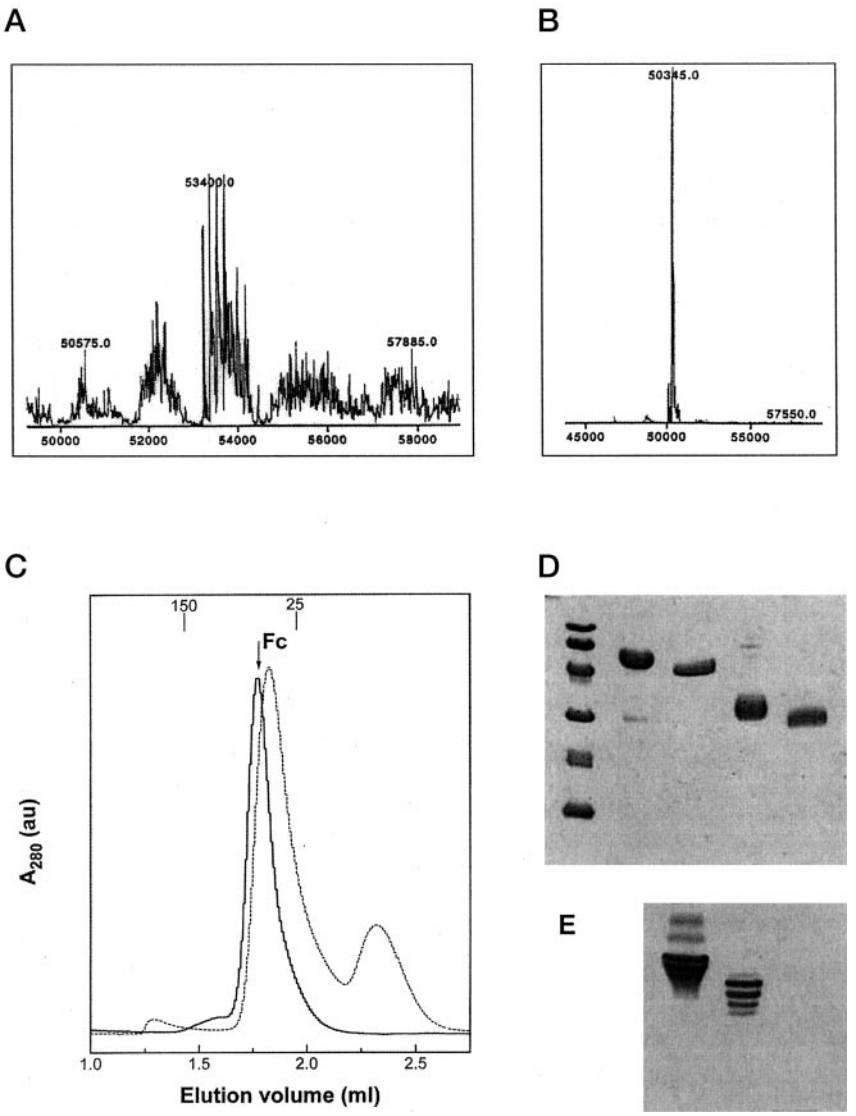

FIG. 1. **Characterization of deglycosylated Fc.** Mass spectrometry measurements of the native Fc (*A*) and the deglycosylated Fc fragment (*B*) of IgG1. The molecular weights (*horizontal axis*) of the respective Fc fragments are labeled. *C*, size exclusion chromatography of the native (*solid line*) and deglycosylated (*dashed line*) Fc. The molecular weight standards are indicated. *D*, SDS-polyacrylamide gel electrophoresis (20% polyacrylamide PhastGel, Amersham Pharmacia Biotech AB) analysis of the native and deglycosylated Fc fragments of IgG1 after digestion with peptide-*N*-glycosidase F (New England BioLabs). *First lane*, molecular weight standard; *second* and *third lanes*, the native and deglycosylated Fc; *fourth* and *fifth lanes*, the native and deglycosylated Fc under reduced conditions. *E*, native gel. *First* and *second lanes*, the native and deglycosylated Fc fragments. The three bands on the native gel of deglycosylated Fc may reflect either conformational heterogeneity or partial degradation.

consistently better than other peptides at all concentrations tested. Due to the low receptor immobilization and the weak binding affinity, the dissociation constant of the receptor-peptide binding could not be derived from these experiments.

Binding of FcγRIII to Immobilized Peptides—To estimate the affinity between the peptides and FcγRIII, individual peptides were immobilized on CM5 sensor chips at 10 mM concentration, and SPR measurements were recorded (Table I). Serial dilutions of the receptor between 750 and 0.37 μM concentrations were used as the analyte (Fig. 4). Among the peptides, the disulfide-linked cIgG1 binds the tightest, with a K_D of 113 μM. This is about 20 times less than the affinity of the native Fc and two time less than that of the deglycosylated IgG1. Among the other peptides, pIgG1, pIgG2, and pIgG4 display similar receptor binding affinity (Table II and Fig. 4). Unexpectedly, pIgE displays significant binding to FcγRIII compared with pALA, although the affinity is 4–10 times lower than other hinge peptides.

Competition between the Peptides and Fc for Receptor Binding—To further investigate whether the peptides share the same receptor binding site as Fc, direct binding competition experiments were performed for the peptides using the native Fc fragment as a competitor. In each experiment, an analyte consisting of 10 μM of FcγRIII mixed with various concentrations of Fc was used to bind to the individual peptides immobilized on CM5 sensor chips. If peptides recognize the same receptor region as that of Fc, the receptor binding to the immobilized peptides will decrease as the concentration of Fc in analyte increases. On the other hand, if the peptides bind to a separate site on the receptor as that of Fc, the SPR response will be independent of or will increase with the Fc concentration due to the higher molecular weight of receptor·Fc complex. The results of competition experiments show that the binding of the receptor to cIgG1, pIgG1, pIgG2, and pIgG4 peptides is blocked by increasing concentrations of Fc (Fig. 5). Because no detectable affinities could be observed between the Fc and the peptides (Table II), the effect of competition resulted directly from the titration of the receptor rather than from the masking of the peptides by Fc. The amount of Fc required to completely block the receptor·peptide interaction is about 10 μM, in agreement with the Fc·receptor binding affinity. This suggests the peptides bind to FcγRIII at the same site as that of Fc. Interestingly, the receptor binding of pIgE displays a similar Fc competition curve as other peptides, indicating that pIgE also shares the same Fc binding site. However, the Fc competition of pIgE is less profound compared with those of other peptides due to a lower receptor·peptide affinity. The control peptide, pALA, displays no measurable receptor binding nor Fc competition.

DISCUSSION

The contribution of glycosylation of Fc to the function of immunoglobulins has been debated over the years. Early stud-

TABLE II
Dissociation constants for the binding of FcγRIII

Immobilization	Analyte	K_D	
		μM	
Binding between the receptor and Fc[a]			
		Flow cell 2	Flow cell 3
FcγRIII	Native Fc	4.6	6.7
	Deglycosylated Fc[b]	>150	>150
	Native IgG1	4.8	3.1
	Deglycosylated IgG1	69.2	43.3
Binding between the receptor and peptides[c]			
cIgG1	FcγRIII	113	
pIgG1		489	
pIgG2		352	
pIgG4		384	
pIgE		1324	
pALA		>4000	
cIgG1	Fc	ND[d]	
pIgG1		ND	
pIgG2		ND	
pIgG4		ND	
pIgE		ND	
pALA		ND	

[a] FcγRIII were immobilized to a CM5 sensor chip in flow cell 2 and 3 via primary amine attachment at approximate concentrations of 15 and 45 μM, respectively. Each binding experiment was carried out with a serial 2× dilution of 8 to 10 concentration points of the analyte starting at 10 μM. The dissociation constant for the binding of the native Fc and IgG1 ($K_D{}^n$) to the CM5 chip was estimated from the steady-state curve fitting. The dissociation of deglycosylated Fc and IgG1 ($K_D{}^d$) was estimated from the equation: $Req^d/Req^n = (K_D{}^n + C)/(K_D{}^d + C)$, where Req and C are equilibrium response unit and the concentration of analyte.

[b] The discrepancy in the K_D of the deglycosylated Fc and IgG is due to a weaker SPR signal generated by Fc fragment compared to that by IgG and a binding approaching to the sensitivity limit of the instrument.

[c] The K_D of peptide binding was obtained by a steady-state curve fitting with the assumptions: 1) the two peptide binding sites on the receptor have equal affinity; 2) only one site on the receptor is occupied by the peptide; 3) the binding follows a first order kinetics.

[d] ND, not detectable.

ies have demonstrated that aglycosylated IgG2a caused a mild reduction in the activation of complement component C1 but a drastic reduction in the activation of Fcγ receptors when compared with the native IgG2a (7, 18). Pound *et al.* (8) found that aglycosylated IgG3 was capable of triggering human phagocyte respiratory burst at 80% of the level triggered by the glycosylated IgG3 despite a severe impairment in antibody-dependent cellular cytotoxicity. Unlike Fcγ receptors, the removal of carbohydrate of IgE did not cause significant loss in FcεRI recognition (9, 19). Structurally, the oligosaccharides attached to Asn^{297} of IgG are a biantennary type with a core heptasaccharide consisting of three *N*-acetylglucosamine (GlcNac) and three manose (Man) and variable fucose additions to the core (6). Unlike most surface-attached glycosylations, these carbohydrates occupy a unique space between the two chains of Fc and appear to have well organized conformations in all crystal structures of Fc with electron densities visible to most of the core sugar moieties. Recently, the crystal structures of IgG1-Fc in complex with FcγRIII and IgE-Fc in complex with FcεRI have been determined (3–5). However, no significant interactions were observed between the carbohydrate on Fc and its receptor in the complex structures. To understand the apparent discrepancy between the known functional importance of this glycosylation and the lack of a structural engagement at the receptor·Fc interface, we have examined the biophysical and binding properties of a deglycosylated IgG1 and its Fc fragment. The Fc fragment binds to FcγRIII at essentially the same affinity as that of an intact IgG1 as measured by BIAcore experiments. Both have a dissociation constant of 4 μM. This agrees well with the previously published ~1 μM for K_D (20, 21). Upon enzymatic deglycosylation, the IgG1-FcγRIII binding

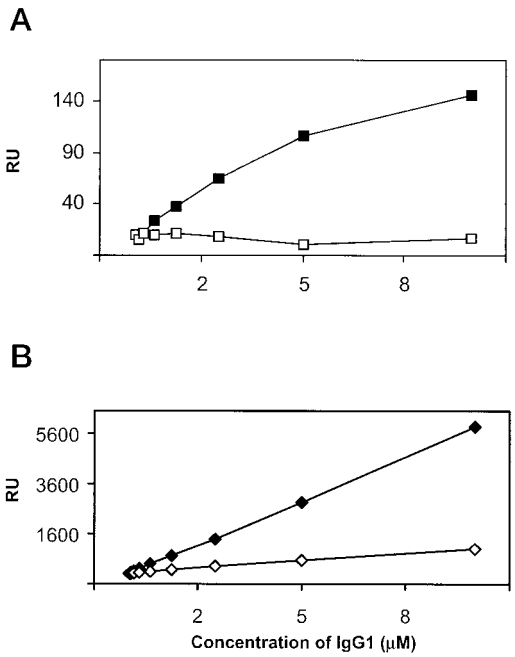

FIG. 2. **Effect of Fc deglycosylation on FcγRIII recognition.** *A*, binding of the native Fc (*solid square*) and deglycosylated Fc (*open square*) to the immobilized receptor. *B*, the binding of IgG1 (*solid diamond*) and its deglycosylated form (*open diamond*) to the receptor. Each data point represents a steady-state SPR response measured in resonance units (RU).

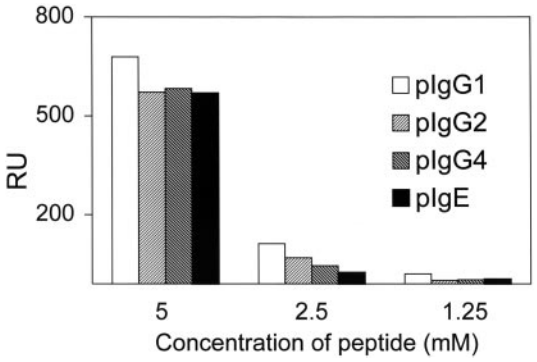

FIG. 3. **Binding of the lower hinge peptides to immobilized FcγRIII.** The three sets of experiments correspond to 5, 2.5, and 1.25 mM peptide concentrations (from *left* to *right*). No detectable binding were observed for pALA at all three concentrations.

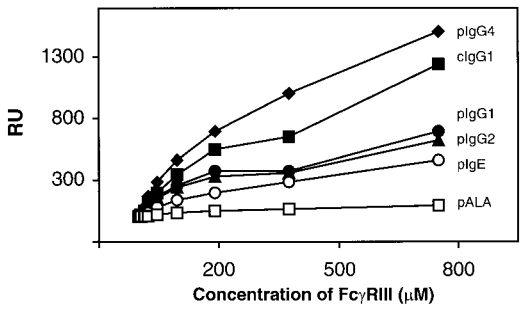

FIG. 4. **FcγRIII binding to peptides immobilized on CM5 sensor chips.** Measurements were performed by using serial dilutions of FcγRIII from 750 to 0.37 μM as analytes. All RUs were normalized against their level of peptide immobilizations using cIgG1 as the standard.

dissociation constant increased from 4 to 50 μM, whereas the Fc·FcγRIII binding became non-detectable. This 10- to 15-fold loss in the receptor binding affinity indicates that the carbohy-

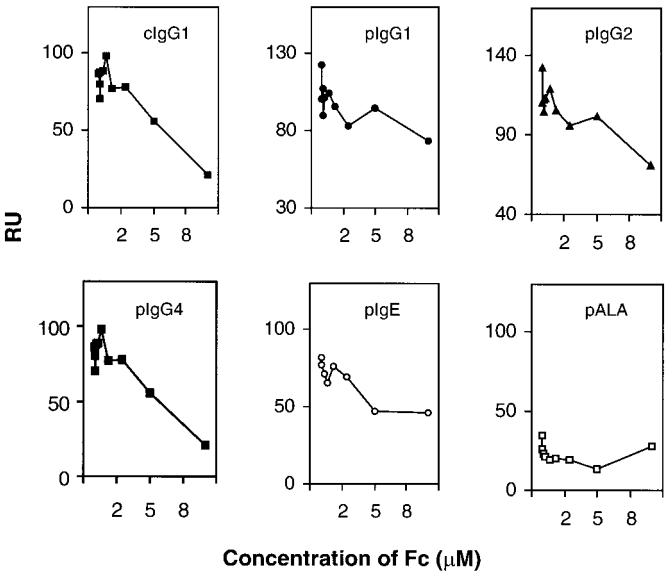

FIG. 5. **Effect of Fc competition on FcγRIII binding to immobilized peptides.** Mixtures of 10 μM FcγRIII with various concentrations of Fc from 10 to 0.02 μM were used as analytes. RU indicates binding of FcγRIII to peptide.

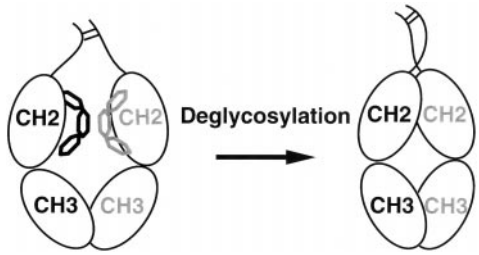

FIG. 6. **Proposed role of glycosylation in stabilizing the Fc conformation.**

drate contributes significantly to the receptor-ligand recognition. Because the sugar moieties make no direct contact with the receptor at the receptor·Fc interface, the most likely role for the carbohydrate is to stabilize the IgG lower hinge in an active receptor binding conformation. The extent of epitope stability provided by the glycosylation is also evident when the affinity of the deglycosylated IgG1 is compared with that of a disulfide-linked peptide, cIgG1. The affinity of the deglycosylated IgG1 is only two times higher than that of cIgG1, which presumably has no defined epitope conformation in solution. It is conceivable that this conformational stability may result from the interaction between the carbohydrate moieties which clearly visible in the electron density of the FcγRIII·Fc complex structure, and may serve as a substitute hydrophobic core. The removal of the carbohydrates by deglycosylation may cause a conformational change in the relative orientation of the two C_H2 domains such that the Fc transitions from an open to a closed conformation (Fig. 6). A structural change associated with aglycosylated IgG3 was previously observed in the vicinity of His^{268} within the C_H2 domain as detected by NMR experiments (7). Analysis of native gel electrophoresis is also consistent with the deglycosylated Fc being more compact than the native Fc (Fig. 1*E*). Alternatively, the carbohydrates may restrict the lower hinge flexibility, and their removal would result in enhanced hinge flexibility and thus reduced receptor binding (22).

It is interesting to note that the deglycosylated Fc consistently resulted in a 2-fold reduction in protein G binding affinity. Even though protein G binds at the C_H2-C_H3 hinge region of Fc, away from the carbohydrate moieties (23), a mild reduction in the protein G binding affinity was observed upon deglycosylation. This suggests a structural change in the C_H2-C_H3 hinge angle as the result of deglycosylation. It is also consistent with the hypothesis that the carbohydrate-free Fc adopts a different hinge conformation.

Besides the normal function of FcR in triggering cellular inflammatory response to clear antigen-bound immune complexes, FcR also mediates autoimmune diseases generated from the response to autoantibodies such as rheumatoid factor in rheumatoid arthritis (12, 13, 24). Under these conditions, it would be beneficial to block the autoantibody-triggered activation of FcR to relieve the auto-inflammatory response that leads to specific tissue damage. Because the activation of FcR requires receptor aggregation by multivalent antigen immune complexes, small molecule compounds that are capable of competing with the binding of immune complexes to FcR should prevent the receptor aggregation and thus inhibit FcR activation (25). The structure of FcγRIII in complex with Fc provides a detailed map of the molecular interface between the receptor and Fc (5). In particular, the dominance of the lower hinge of Fc, which occupies 60% of the interface area, suggests that peptides resembling the lower hinge conformation could be good candidates to inhibit the receptor function. In an attempt to evaluate such peptide inhibitors and their ability to compete with the receptor binding to the native ligand, we designed four peptides with sequences encompassing the receptor binding region of the lower hinges of IgG1, -2, -4, and IgE. Among these peptides, the disulfide-linked IgG1 hinge peptide, cIgG1, binds the tightest to FcγRIII with an affinity ~20 times less than the native immunoglobulins and three times better than the non-disulfide-linked peptide, pIgG1 (Table II). The observed difference in receptor binding between cIgG1 and pIgG1 suggests that the disulfide bond located at the lower hinge region of Fc contributes to its conformational stability. The three IgG-derived peptides bind to the receptor with approximately the same affinity. An unexpected result is that the IgE-derived peptide, pIgE, possesses a significant binding affinity to FcγRIII. This is particularly interesting, because the lower hinge sequence of IgE is quite different from those of IgGs. It suggests that the binding of low affinity receptors may be quite promiscuous. It leads to the possibility of FcγRIII activation by antigen-bound IgE under certain circumstances such as in a saturated allergen condition or in the absence of Fcε receptors. In fact, the binding of IgE-immune complexes to the low affinity Fcγ receptors on mast cells has been observed to trigger the release of serotonin (26).

The competition results show that all lower hinge peptides compete directly with Fc in receptor binding. The ability of these lower hinge peptides to inhibit Fc binding to the receptor opens potential new ways of designing therapeutic compounds. For examples, pIgG analogs may be useful in treating Fcγ receptor-mediated autoimmune diseases by blocking the activation of the receptor, or pIgE-like compounds could be used as potent inhibitors of Fcε receptors thus providing a potential treatment for allergy.

This study on the binding affinity of the lower hinge peptides has also allowed us to examine the issue of the receptor isotype specificity. The low affinity human FcγRIII binds to IgG1 and IgG3 much better than it does to IgG2 and IgG4 (16). Previous mutation studies of IgG2 and its binding to human high affinity receptor FcγRI allow us to conclude that the entire lower hinge sequence was required to restore the IgG1 binding affinity in IgG2, whereas point mutations in IgG1 hinge residues resulted in a loss of the receptor binding (15). In this work, the lower hinge peptides instead of the antibodies were used in the study of the receptor binding. This enables us to separate the indi-

vidual amino acid contribution to the receptor affinity from the effect of their environment, namely the length of lower hinge in an intact antibody. The results from studies of solution binding between the receptor and immobilized peptides and from Fc competition assays show that pIgG2 and pIgG4 have nearly the same affinity to FcγRIII as does pIgG1. Replacing Leu with Phe in pIgG4 or changing Glu-Leu-Leu-Gly to Pro-Val-Ala in pIgG2 in addition to a single residue deletion makes little difference in the affinity toward the receptor. This suggests that factors other than the lower hinge amino acid composition play an important role in determining the weaker binding affinity of IgG2 and IgG4 to FcγRIII (relative to IgG1). It has been proposed that the overall length of the lower hinge may be important to the receptor IgG subtype specificity (16), because the hinges of IgG1 and -3 are about three residues longer than those of IgG2 and -4. It is also possible, however, that each IgG subtype varies in its glycosylation at Asn^{297} and that the differences in carbohydrate may contribute to the observed receptor specificity. Some preference in glycosylation of IgGs is known to exist (6). Residues outside the lower hinge region but in the vicinity of receptor interface could also influence the receptor binding preference. For example, Pro^{329} of Fc is sandwiched between Trp^{90} and Trp^{113} of the receptor, providing important van der Waals contacts between the receptor and Fc. Although Pro^{329} is conserved among the IgG subtypes, residue 327 displays a dimorphism with an Ala in IgG1 and -3 and a Gly in IgG2 and -4.

In conclusion, the present study suggests that glycosylation at Asn^{297} of Fc fragments plays an important role in the binding of Fc fragments to the low affinity receptor FcγRIII. The role of carbohydrate appears to be primarily to stabilize the receptor epitope conformation. We also demonstrated that small peptide ligands can be designed to inhibit the binding of FcγRIII to its natural ligand Fc.

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